

# Effects of postharvest storage and dormancy status on ABA content, metabolism, and expression of genes involved in ABA biosynthesis and metabolism in potato tuber tissues

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**Abstract** At harvest, and for an indeterminate period thereafter, potato tubers will not sprout and are physiologically dormant. Absciscic acid (ABA) has been shown to play a critical role in tuber dormancy control but the mechanisms controlling ABA content during dormancy as well as the sites of ABA synthesis and catabolism are unknown. As a first step in defining the sites of synthesis and cognate processes regulating ABA turnover during storage and dormancy progression, gene sequences encoding the ABA biosynthetic enzymes zeaxanthin epoxidase (ZEP) and 9-*cis*-epoxycarotenoid dioxygenase (NCED) and three catabolism-related genes were used to quantify changes in their relative mRNA abundances in three specific tuber tissues (meristems, their surrounding periderm and underlying cortex) by qRT-PCR. During storage, *StZEP* expression was relatively constant in meristems, exhibited a biphasic pattern in periderm with transient increases during early and mid-to-late-storage, and peaked during mid-storage in cortex. Expression of two members of the potato NCED gene family was found to correlate with changes in ABA content in meristems (*StNCED2*) and cortex (*StNCED1*). Conversely, expression patterns of three putative ABA-8'-hydroxylase (*CYP707A*) genes during storage varied in a tissue-specific manner

with expression of two of these genes rising in meristems and periderm and declining in cortex during storage. These results suggest that ABA synthesis and metabolism occur in all tuber tissues examined and that tuber ABA content during dormancy is the result of a balance of synthesis and metabolism that increasingly favors catabolism as dormancy ends and may be controlled at the level of *StNCED* and *StCYP707A* gene activities

**Keywords** ABA · Dormancy · Gene expression · Potato · *Solanum tuberosum* L. · Tuber · qRT-PCR

## Abbreviations

ABA Absciscic acid  
NCED 9-*cis*-epoxycarotenoid dioxygenase  
ZEP Zeaxanthin epoxidase

## Introduction

The fundamental mechanisms controlling dormancy in the potato tuber remain enigmatic. However, recently an intriguing and complex picture has started to emerge. It is now generally accepted that tuber dormancy is initiated concurrently with tuber initiation (Burton 1989; Vreugdenhil 2004). The inheritance pattern of tuber dormancy is complex, and QTL analyses have indicated that tuber dormancy is controlled by at least nine different loci (Van den Berg et al. 1996; Ewing et al. 2004).

At the physiological level, plant hormones have been shown to be intimately involved in potato tuber dormancy control (Classens and Vreugdenhil 2000; Suttle 2004). In particular, sustained synthesis and action of endogenous

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abscisic acid (ABA) is required for both the initiation and maintenance of tuber dormancy (Suttle and Hultstrand 1994). ABA is actively metabolized in tuber tissues throughout dormancy and, in general, tuber ABA content declines during storage (Suttle 1995; Biemelt et al. 2000).

ABA content is influenced by an array of developmental and environmental cues and interplay with other phytohormones (Nambara and Marion-Poll 2005). ABA is synthesized from the C<sub>40</sub> carotenoid zeaxanthin by a series of enzymatic steps (Xiong and Zhu 2003). Genes encoding most enzymes of this pathway have been identified and cloned from different plant species (Nambara and Marion-Poll 2005). The first two steps of this pathway occur in the plastids (Xiong and Zhu 2003). These initial reactions are catalyzed by zeaxanthin epoxidase (ZEP), which converts zeaxanthin to the di-epoxycarotenoid violaxanthin via the mono-epoxy, antheraxanthin. Violaxanthin is further oxidized to neoxanthin by an incompletely understood mechanism. Following isomerization, the *cis*-isomers of violaxanthin and/or neoxanthin are cleaved to a C<sub>15</sub> product, xanthoxin, and a C<sub>25</sub> metabolite by 9-*cis*-epoxycarotenoid dioxygenase (NCED) which is the first committed step of the ABA pathway (Nambara and Marion-Poll 2005). In all plant species examined thus far, NCED genes belong to a multi-gene family. In *Arabidopsis*, sequence and functional analyses have indicated that five of nine NCED-related sequences are involved in ABA biosynthesis (Tan et al. 2003; Lefebvre et al. 2006). Xanthoxin is exported to the cytosol, where it is converted to ABA via ABA-aldehyde in two enzymatic steps. To date, genes encoding these final two enzymes have been characterized only in *Arabidopsis*. A short-chain alcohol dehydrogenase/reductase (SDR) encoded by the single-copy *AtABA2* gene catalyzes the conversion of xanthoxin to ABA aldehyde (Cheng et al. 2002; González-Guzman et al. 2002). Oxidation of ABA-aldehyde to ABA is catalyzed by abscisic aldehyde oxidase. Although the *Arabidopsis* genome has four aldehyde oxidase genes (AAO), identification of null *AtAAO3* alleles exhibiting severe ABA-deficient phenotypes in seeds suggests that *AtAAO3* is the principal gene involved in ABA synthesis in that tissue (González-Guzman et al. 2004; Seo et al. 2004).

Metabolism of ABA can occur by reversible conjugation with glucose or irreversible oxidation at either the 7', 8', or 9'-methyl group (Zhou et al. 2004; Nambara and Marion-Poll 2005). In most plant tissues including potato tubers (Suttle 1995), the predominant ABA catabolic pathway begins with hydroxylation of the C-8'-methyl group to form the unstable intermediate, 8'-hydroxy ABA which spontaneously rearranges to phaseic acid (PA) (Cutler and Krocho 1999). ABA 8'-hydroxylase has been extensively characterized in vitro and shown to be a membrane-asso-

ciated cytochrome P450 mono-oxygenase (Krochko et al. 1998). Recently, four *Arabidopsis* P450 *CYP707A* genes were shown to encode ABA 8'-hydroxylases (Kushiro et al. 2004; Saito et al. 2004). *CYP707A*-related sequences have also been found in the rice genome and among EST's from tomato, soybean, corn, lettuce, and wheat (Nambara and Marion-Poll 2005).

Although the role of ABA in tuber dormancy has been established, the sites of ABA synthesis and catabolism in tubers are unknown. As a first step in defining the sites of synthesis and cognate processes regulating ABA turnover during postharvest storage and dormancy progression, gene sequences encoding the potentially rate-limiting ABA biosynthetic enzymes ZEP and NCED and three *CYP707A*-related genes have been used to monitor expression patterns in three specific tuber tissues (meristem, periderm, cortex) by qRT-PCR. In general, expression of the biosynthetic genes was observed to moderate towards the end of dormancy whereas expression of the *CYP707A*-related catabolic genes increased during storage in a differential manner. The results are in agreement with parallel determinations of tuber tissue ABA content and ABA metabolism during storage and dormancy progression. The results suggest that the sustained ABA synthesis required for tuber dormancy maintenance is the result of a dynamic equilibrium of ABA biosynthesis and degradation that progressively favors degradation as dormancy progresses.

## Materials and methods

### Plant material

Field-inspected and certified, seed potato (*Solanum tuberosum* L. cv Russet Burbank) tubers were grown in northwest Minnesota under standard cultural conditions. After harvest in late September, tubers were allowed to undergo skin set at 20°C for 2 weeks and thereafter stored at 3°C (ca. 95% RH) in the dark. Tubers were transferred to room temperature, hand washed, and stored in the dark for 3 days prior to tissue isolation. Primary meristems (buds) were isolated from eyes using a 1 mm curette with the aid of a dissecting microscope (Law and Suttle 2002). Subsequently, a core borer (#4) was used to collect a 7 mm in diameter and approximately 15 mm long cylinder of periderm and cortex surrounding the excised meristem. Periderm tissue (1 mm thick) and 5 mm of underlying cortex were quickly removed from the cored cylinder. All tissues were frozen in liquid N<sub>2</sub> immediately after excision and were stored at -80°C until further use. All studies reported in this paper utilized simultaneously harvested batches of tissue samples.

## ABA extraction and analysis

Groups of 100 meristems (0.1–0.14 g FW), five periderm discs (0.1–0.24 g FW) or three cortex cylinders (0.6–0.9 g FW) were allowed to thaw at 4°C in 80% (v/v) aqueous acetone. The tissue was mechanically homogenized (4°C), clarified by centrifugation (10,000g for 15 min), and the supernatant decanted. The pellet was re-extracted in 80% (v/v) aqueous acetone and, after standing for  $\geq 2$  h (4°C), was re-centrifuged and the supernatants combined. Depending on the sample, a total of 50–100 ng  $^2\text{H}_6$ (+)-ABA (OIChemIm Ltd, Olomouc, Czech Republic<sup>1</sup>) was added as an internal standard. The supernatants were dried under a stream of  $\text{N}_2$  (40°C) and re-dissolved in 5 ml 1 M formic acid. The acidified extracts were loaded onto a 150 mg MCX Oasis cartridge (Waters Associates, Milford, MA). The cartridge was washed with 5 ml 1 M formic acid followed by 5 ml methanol. The methanol fraction was taken to dryness under a stream of  $\text{N}_2$  (40°C) and was re-dissolved in 10 mM ammonium acetate (pH 5.6). ABA was quantified by high performance liquid chromatography–mass spectrometry (HPLC–MS) using a Thermo Electron Surveyor MSQ system and a 2.1×150 mm 5  $\mu\text{m}$  Hypersil Gold Column (Thermo-Finnigan, San Jose, CA). HPLC solvents were: A, 10 mM ammonium acetate (pH 5.6) and B, methanol (0.2 ml/min). Starting conditions were 40% B, a linear gradient to 80% B in 5 min followed by a linear gradient to 100% B in 5 min. Detection and quantification were performed using the MSQ operating in the negative ion, electrospray ionization mode with a probe temperature of 460°C, cone voltage of 30 V, and needle voltage of 4.5 kV. Ions ( $m/z$ ) monitored were: 263 for ABA and 269 for [ $^2\text{H}$ ]<sub>6</sub>-ABA. Each sample replicate was injected at least twice.

## ABA metabolism studies

Meristems were isolated with a curette and were briefly washed in deionized water followed by incubation buffer (10 mM MES-KOH, pH 5.7) at room temperature. Groups of 10 meristems were incubated in 1 ml of buffer containing 37 kBq (0.1 nmol) [ $^3\text{H}$ ]( $\pm$ )-ABA (550 GBq/mmol; American Radiochemicals, Inc., St Louis, MO) and were incubated at room temperature with constant agitation. After 4 h, the meristems were removed, washed extensively with running deionized water, blotted dry, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$ . Meristems were mechanically homogenized in 80% (v/v) aqueous acetone (4°C) and clarified by centrifugation (10,000g for 15 min).

<sup>1</sup> Mention of a trademark or proprietary product does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

The supernatants were taken to dryness under a stream of nitrogen (40°C) and re-dissolved in 1% [v/v] acetic acid. Extracts were fractionated by reverse-phase HPLC coupled with an in-line radioactivity monitor as described previously (Suttle 1995). Metabolite identification was achieved by co-chromatography with authentic standards (Suttle 1995).

## RNA extraction

To isolate total RNA from meristems (100 eyes), TRIZOL Reagent (Invitrogen, Carlsbad, CA) was used following the manufacturer's recommendations. Total RNA from periderm (20 discs) and cortical (five cylinders) tissues was isolated according to Narváez-Vásquez and Ryan (2002) with modifications. Briefly, ca. 0.6–0.8 g (FW) of ground tissue was extracted with a mixture of 500  $\mu\text{l}$  of 0.1 M Tris–HCl (pH 7.4) containing 1% (w/v) sodium sulfite and 500  $\mu\text{l}$  of water-saturated phenol and the extracts were clarified by centrifugation (14,000g for 10 min). The supernatants were re-extracted twice with an equal volume of acid-phenol/chloroform (5:1 v/v, Ambion, Austin, TX) followed by two extractions with an equal volume of chloroform. The RNA was precipitated with an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at  $-20^\circ\text{C}$  overnight. RNA quality was determined by agarose gel electrophoresis in 1× TBE followed by ethidium bromide staining and UV light visualization. Before analyzing for specific mRNAs, total RNA (10–15  $\mu\text{g}$ ) was treated with DNA-free (Ambion) to eliminate genomic DNA contamination.

## qRT-PCR analysis

Total RNA (1.5  $\mu\text{g}$ ) was reverse transcribed using the RETROscript kit (Ambion), with oligo dT<sub>18</sub> as the primer, according to the manufacturer's recommendations. The cDNA was then diluted in a total volume of 170  $\mu\text{l}$  with sterile RNase-free water. Amplification of specific regions of targeted genes and real-time detection of amplicon production was conducted using a DNA Engine Opticon 2 (BioRad, Hercules, CA). The sequences of the primer pairs used for each gene are presented in Table 1. Reactions contained 2  $\mu\text{l}$  of primer mix (containing 0.5  $\mu\text{M}$  of each forward and reverse primer), 5  $\mu\text{l}$  cDNA template (derived from 8.82 ng/ $\mu\text{l}$  total RNA), 6.1  $\mu\text{l}$  SYBR-Green master PCR mix containing 2  $\mu\text{l}$  10× SYBR-Green Taq Buffer (100 mM Tris pH 8.5, 500 mM KCl, 1.5% Triton X-100), 1.6  $\mu\text{l}$  25 mM  $\text{MgCl}_2$ , 0.4  $\mu\text{l}$  10 mM dNTPs, 1  $\mu\text{l}$  DMSO, 1  $\mu\text{l}$  SYBR-Green stock, 0.1  $\mu\text{l}$  (2.5 units/ $\mu\text{l}$ ) Taq Polymerase (Promega, Madison, WI), and 6.9  $\mu\text{l}$  water to make a total volume of 20  $\mu\text{l}$ . SYBR-Green 10× stock was

**Table 1** *Solanum tuberosum* primer sequences used in this study

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
<i>StZEP</i>	TATCTGAGAAAGCAAATGACC	GTAGGGAAGTTTGGAGACGT
<i>StNCED1</i>	GGAAATCAACAAGAAAAGCCA	ATATTTGTTGTCACCATAAATGAA
<i>StNCED2</i>	GGGACTTTCATTAGCTCAAAGGACTTGC	GCGATGTAAATTTGAATTACTATTATTCGCTCA
<i>StCYP707A1</i>	GTACAGGTGGTCTATGGTGG	CGATGCTTGTGTCTTGATGTTGATTG
<i>StCYP707A2</i>	TGAGTGTGTCAAGGCTGAACAGAAAGTT	GAATGCCACTACCAGATCCTACCCTTC
<i>StCYP707A3</i>	TGTGTTTAAGCTCTTTGATTGTGGTG	TGCTCGAAGAACTAGCTTTCTTAGC
<i>StCCD</i>	GAGGATGGACATGATACATGC	GACATGAGCCACAATGTCAAG

prepared in TE buffer (pH 7.5) from a 10,000× concentrate (FMC Bioproducts, Rockland, ME) frozen at  $-20^{\circ}\text{C}$  and thawed only once before use. Reactions were carried out under the following conditions:  $94^{\circ}\text{C}/2$  min (1 cycle);  $58^{\circ}\text{C}/1$  min (1 cycle);  $72^{\circ}\text{C}/1$  min (1 cycle);  $94^{\circ}\text{C}/30$  s,  $58^{\circ}\text{C}/30$  s;  $72^{\circ}\text{C}/45$  s (35 cycles).

PCR amplification of a single product of the correct size for each gene was confirmed by agarose gel electrophoresis and double-strand sequencing. The amplified fragment of each gene was subcloned and used to generate efficiency curves. Relative fold expression for each gene was calculated by the method of Pfaffl (2001). The transcript of *StCCD*, a possible orthologue of *AtCDD4*, was used to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations.

GenBank accession numbers of the potato genes used in this study are: *StZEP* (DQ206629), *StNCED1* (AY662342), *StNCED2* (AY662343), *StCYP707A1* (DQ206630), *StCYP707A2* (DQ206631), *StCYP707A3* (DQ206632), and *StCCD* (DQ206633).

### Experimental design

The studies described in this paper were conducted over a 3-year period that included two separate harvest/storage seasons. The absolute length of tuber dormancy varies from year to year in an unpredictable manner (Burton 1989). Because of this, data from multiple years cannot be combined and averaged. Nevertheless, the results obtained during these studies were qualitatively similar. For these reasons, only data from the 2004 to 2005 harvest and storage season are presented.

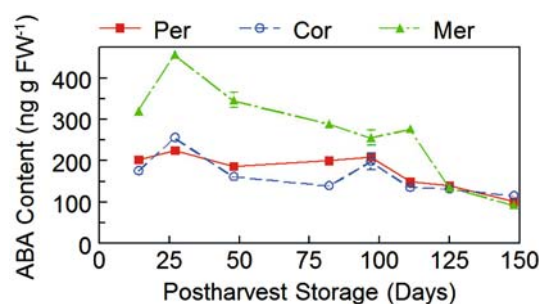
### Results

After harvest and wound-healing, the tubers used in these studies were stored in the dark at  $3^{\circ}\text{C}$  (95% RH). Periodically thereafter, tubers were transferred to  $20^{\circ}\text{C}$  for 3 days prior to use. Under these conditions, the tubers were completely dormant (i.e., no sprout growth after 2 weeks at  $20^{\circ}\text{C}$ ) for at least 48 days of storage. Thereafter, dormancy weakened and by 97 days of storage tubers exhibited

limited ( $<5$  mm) sprout growth 2 weeks after transfer to  $20^{\circ}\text{C}$ . After 125 days of storage, tubers were completely non-dormant and exhibited vigorous (5–15 mm) sprout growth after transfer to  $20^{\circ}\text{C}$ .

### Changes in ABA content during physiological dormancy progression

Initially, tissue-specific changes in endogenous ABA content during postharvest storage were determined. The tissues analyzed included meristems of dormant tubers and their surrounding periderm and underlying cortical tissue. Little change in ABA content was observed in periderm tissue up to 100 days followed by a smooth decline over the next 50 days (Fig. 1). In contrast, the ABA content of meristems rose 43% during the first 27 days of storage then fell steadily until 97 days. At 111 days there was a slight but statistically significant increase in meristem ABA content followed by a sharp decline to 125 days and a more gradual decline thereafter. Similarly, in cortical tissue there was a 45% increase in ABA content at 27 days and then a steady decrease up to 82 days. At 97 days there was a moderate but significant rise followed by a continuous drop up to 148 days.



**Fig. 1** Effects of postharvest storage on endogenous levels of free ABA in field-grown Russet Burbank tubers (storage season 2004–2005). At the indicated times, tubers were transferred from 3 to  $20^{\circ}\text{C}$  3 days prior to tissue isolation. Tissue samples were taken from meristems ( $\blacktriangle$ ), underlying cortex ( $\circ$ ) and surrounding periderm ( $\blacksquare$ ) and stored at  $-80^{\circ}\text{C}$ . ABA was quantified by HPLC–MS as described in Material and methods. Values presented are means  $\pm$  SE ( $n=3$ )

## Postharvest storage effects on the ABA metabolism in meristems of potato tubers

The marked decline in endogenous ABA content in meristems during dormancy progression suggested an increase in ABA metabolism towards the end of dormancy. To examine this possibility further, the metabolism of exogenous [ $^3\text{H}$ ]-( $\pm$ )-ABA in meristems was examined as a function of postharvest storage duration. At all harvest dates, exogenous [ $^3\text{H}$ ]-( $\pm$ )-ABA was readily metabolized to two main products (phaseic acid [PA] and dihydrophaseic acid [DPA]) by tuber meristem tissues (Table 2). In highly dormant tubers ( $\leq 40$  days of storage), 84% of the recovered radioactivity was associated with unaltered ABA after 4 h of incubation and the remaining 16% was associated with PA and its metabolite DPA. This rate of metabolism remained unchanged for a further 50 days of storage. As storage was extended to 132 days, the overall rate of ABA metabolism increased nearly two-fold with 28% of the radioactivity distributed equally between PA and DPA. After 142 days of storage there was a further increase in the rate of ABA metabolism. In this case, the majority of radioactivity was associated with DPA and an unknown metabolite tentatively identified as the glucose ester of DPA.

## Characterization of ABA metabolic genes of potato

A search of the TIGR tomato and potato gene index (<http://www.tigr.org>) revealed several tentative consensus sequences and ESTs with varying degrees of homology to all characterized ABA biosynthetic genes. Using these sequences, our group has isolated the potato orthologues: *StZEP*, *StNCED1*, a new member of the NCED gene family, *StNCED2* and three members of the potato *CYP707A* gene family.

*StZEP* (GeneBank Accession DQ206629) is a 2046 bp cDNA that encodes a protein of 670 amino acids with a predicted molecular weight of 73.1 kD and a *pI* of 6.36. BLAST analysis indicated that *StZEP* shows very high sequence homologies to ZEP proteins from other plant

species including tomato, *Nicotiana plumbaginifolia*, *Arabidopsis*, grapes, pepper, and cowpea (Supplementary Material Fig. 1). Analysis of the predicted protein indicated that, as other ZEP proteins, it has a N-terminal chloroplast transit peptide, a mono-oxygenase domain, and a FHA motif of unknown function.

*StNCED1* (GeneBank Accession AY662342) is a 2300 bp cDNA that contains a single open reading frame encoding a protein of 603 amino acids with a calculated relative mass of 67.1 kD and a *pI* of 6.16. BLAST analysis of the deduced potato protein sequence indicated a 96% identity and a 98% similarity with the *LeNCED1* protein (Burbidge et al. 1997).

*StNCED2* (GeneBank Accession AY662343) is a 2143 bp cDNA. The single 1743 bp open reading frame encodes a protein of 580 amino acids with a calculated mass of 64.7 kD and a *pI* of 6.21. BLAST analysis of the predicted protein revealed extensive homology with NCED proteins from other plants including *VvNCED1* and *VvNCED2* from grapes (73 and 74% identity), *LsNCED1* from lettuce (74% identity), *LeNCED1* from tomato (72% identity) and *StNCED1* from potato (71% identity). This high degree of homology, suggests that both *StNCEDs* likely encode 11, 12 (11', 12')-NCED activities (Supplementary Material Fig. 2). Furthermore, both *StNCEDs* contain the four highly conserved histidine residues ( $\text{H}_{172}$ ,  $\text{H}_{349}$ ,  $\text{H}_{414}$ ,  $\text{H}_{590}$  for *StNCED1* and  $\text{H}_{150}$ ,  $\text{H}_{327}$ ,  $\text{H}_{392}$ ,  $\text{H}_{567}$  for *StNCED2*) found in other NCEDs, putative plastid-targeting transit peptides, and an amphipatic sequence (92–109 for *StNCED1* and 70–87 for *StNCED2*) present in all *Arabidopsis* NCEDs and required for membrane binding in maize VP14 (*ZmNCED1*) (Schwartz et al. 1997; Tan et al. 2003).

*StCYP707A's* (GeneBank Accessions DQ206630, DQ206631 and DQ206632) are two full-length and one partial cDNAs which encode three putative P450 mono-oxygenases exhibiting very high sequence homologies to all four members of the *Arabidopsis thaliana* *CYP707A* gene family (Supplementary Material Fig. 3). At the amino acid level, the predicted potato proteins are 61–80% identical to *AtCYP707A1*, *AtCYP707A3*, *AtCYP707A4* and *AtCYP707A2* in that order. Other proteins with significant homologies are *didiG2* from tobacco and several uncharacterized proteins from rice. A comprehensive molecular and biochemical study of these three members of the potato *CYP707A* gene family will be published elsewhere.

## Expression of the ABA biosynthetic pathway during tuber dormancy progression

To examine the expression of ABA biosynthetic and catabolic genes during potato tuber dormancy, real time PCR

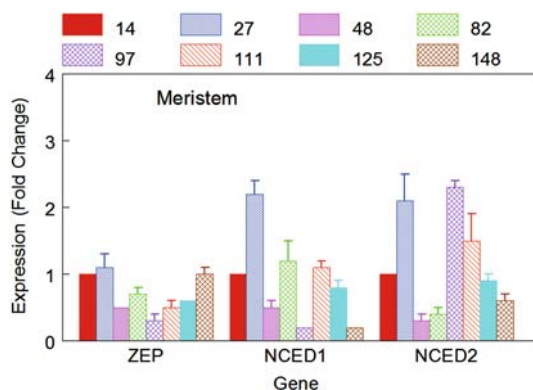
**Table 2** Effects of postharvest storage on metabolism of [ $^3\text{H}$ ]-( $\pm$ )-ABA in isolated tuber meristems. Meristems were isolated from tubers after various periods of storage and were floated on solutions of [ $^3\text{H}$ ]-( $\pm$ )-ABA in buffer for 4 h

Postharvest storage (days)	Radioactivity (% recovered)			
	Unknown	DPA	PA	ABA
33	0	6 $\pm$ 0	10 $\pm$ 1	84 $\pm$ 0
40	0	8 $\pm$ 2	8 $\pm$ 0	84 $\pm$ 2
91	0	9 $\pm$ 1	10 $\pm$ 1	81 $\pm$ 0
132	0	16 $\pm$ 2	13 $\pm$ 2	72 $\pm$ 1
142	5 $\pm$ 3 <sup>a</sup>	32 $\pm$ 1	1 $\pm$ 0	62 $\pm$ 3

<sup>a</sup> Mean  $\pm$  SD

was performed with RNA isolated from tuber meristems, periderm and cortex. Relative fold expression for each gene was calculated by the method of Pfaffl (2001). Preliminary experiments demonstrated that expression of a *StCCD* gene was constant during tuber storage and it was therefore used as the reference gene to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations (Supplementary Material Fig. 4). The initial time point of the storage season was designated as control and set equal to one. Using un-normalized control data (in triplicate) and experimentally determined efficiency curves for each gene, the approximate number of transcripts (per  $\mu\text{g}$  total RNA) in the three different tissues was calculated. Transcript copy number varied considerably among genes and tissues (Table 3). In all three tissues, the transcript abundances of *StNCED1* were considerably lower than other genes encoding ABA biosynthetic enzymes. Interestingly, the copy number for all ABA metabolic genes was highest in meristems and lowest in cortex.

The relative expression of *StZEP*, *StNCED1*, and *StNCED2* in meristems during storage is shown in Fig. 2. Expression of the *StZEP* gene exhibited little overall change during storage: remaining nearly constant during the first 27 days of storage, declining to a minimum after 97 days and returning to its initial level at 148 days of storage. Expression analysis of the two NCED gene members examined in this study revealed a similar early transient increase after 27 days of storage. Thereafter, expression of *StNCED1* fell after 48 days, rose at 82 days, fell to a minimum after 97 days of storage, rose again after 111 days, and then fell continuously as storage was extended to 148 days. The expression pattern of *StNCED2* was biphasic. After the initial rise, *StNCED2* expression

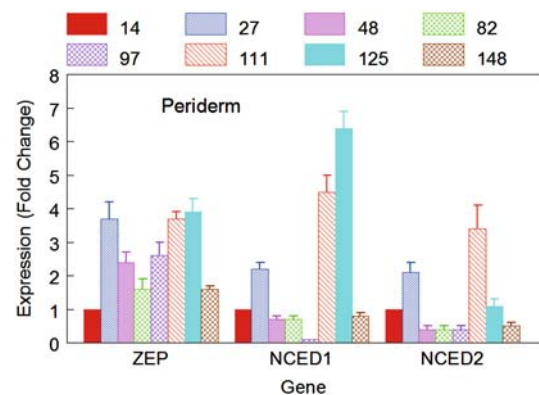


**Fig. 2** Quantitative expression analysis of ABA biosynthetic genes in tuber meristems during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from lateral meristems. Relative fold expression for each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. Expression for each gene is presented as fold change relative to the initial time point of the storage season. Each gene was analyzed in triplicate. Values presented are means  $\pm$  SE ( $n = 3$ )

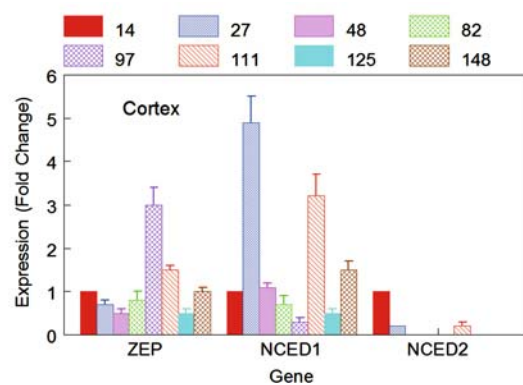
fell to a minimum between 48 and 82 days of storage, rose again to a second maximum by 97 days, and fell thereafter.

Gene expression analysis of the ABA biosynthetic machinery in periderm is presented in Fig. 3. *StZEP* expression was biphasic exhibiting a transient maximum after 27 days of storage, a decline until 82 days, a second peak between 111 and 125 days of storage, and a final decline at 148 days. *StNCED1* and *StNCED2* expression patterns were similar, with transient increases at 27 days and maximum expression at 125 days for *StNCED1* and at 111 days for *StNCED2*.

Expression of these genes in cortex displayed a rather different pattern (Fig. 4). *StZEP* expression remained



**Fig. 3** Quantitative expression analysis of ABA biosynthetic genes in periderm during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from periderm tissues. Relative expression of each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. Expression of each gene is presented as fold change relative to the initial time point of the storage season. Values presented are means  $\pm$  SE ( $n = 3$ )



**Fig. 4** Quantitative expression analysis of ABA biosynthetic genes in cortex during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from cortex tissue. Relative fold expression of each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. Expression of each gene is presented as fold change relative to the initial time point of the storage season. Values presented are means  $\pm$  SE ( $n = 3$ )



**Table 3** Estimated abundances of ABA biosynthetic and catabolic gene transcripts in potato tissues at the start of storage

Gene	Copy number/ $\mu$ g total RNA		
	Meristems <sup>a</sup>	Periderm	Cortex
<i>StZEP</i>	$9.63 \pm 0.97 \times 10^6$	$1.29 \pm 0.17 \times 10^6$	$1.26 \pm 0.66 \times 10^5$
<i>StNCED1</i>	$5.82 \pm 0.48 \times 10^5$	$7.91 \pm 1.61 \times 10^4$	$3.92 \pm 1.05 \times 10^2$
<i>StNCED2</i>	$1.90 \pm 0.07 \times 10^7$	$9.71 \pm 0.91 \times 10^6$	$2.54 \pm 0.23 \times 10^6$
<i>StCYP707A1</i>	$7.45 \pm 1.21 \times 10^4$	ND <sup>b</sup>	$3.10 \pm 0.37 \times 10^4$
<i>StCYP707A2</i>	$6.16 \pm 1.18 \times 10^5$	$7.03 \pm 1.34 \times 10^4$	$1.43 \pm 0.14 \times 10^5$
<i>StCYP707A3</i>	$2.37 \pm 0.17 \times 10^5$	$9.36 \pm 0.33 \times 10^4$	ND

<sup>a</sup> Mean  $\pm$  SEM ( $n = 3$ )<sup>b</sup> ND: not detected

nearly constant for the first half of storage before rising to a maximum after 97 days, respectively. Expression of *StNCED1* was biphasic and displayed two maxima at 27 and 111 days of storage. Expression of *StNCED2* was highest at 14 days, declined sharply to near-undetectable levels during mid-storage (48–97 days), rose slightly at 111 days and declined below the limits of detection for the remainder of the study.

#### Expression of genes involved in ABA catabolism during physiological dormancy progression

Analysis of the three *StCYP707A* genes examined in this study revealed a differential expression pattern not only across the tissues examined but among themselves. In meristems, the overall expression of *StCYP707A1* and

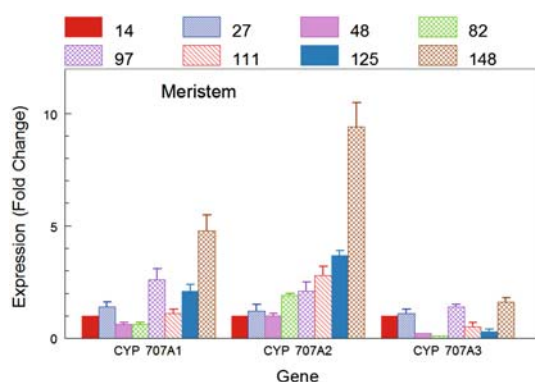
*StCYP707A2* increased during dormancy progression (Fig. 5). In contrast, *StCYP707A3* expression remained at or below initial levels across storage but exhibited transient maxima during early, mid- and late-storage. In periderm, only transcripts of *StCYP707A2* and *StCYP707A3* were detected; mRNA of *StCYP707A1* was absent or below the limits of detection. In general, expression of *StCYP707A2* and *StCYP707A3* was low during the first 97 days of storage but rose dramatically (8–60-fold) by 125 and 111 days, respectively. By 148 days, expression of both genes declined especially *StCYP707A3* which was below the limits of detection.

In marked contrast with the periderm, only *StCYP707A1* and *StCYP707A2* transcripts were detected in cortex and, their expression was highest at the start of storage (14 days) and remained very low throughout storage (Fig. 6). Transcripts of both genes exhibited a modest rise after 111 days of storage to levels well below the initial values. With the exception of one time point (day 97), expression of *StCYP707A3* was below the limits of detection.

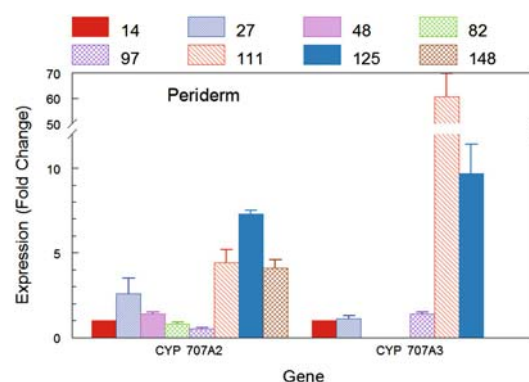
## Discussion

### Endogenous ABA content changes during potato dormancy progression

The life cycle of the potato tuber has been divided into five distinct stages: tuber induction, initiation, enlargement, dormancy, and sprouting (Fernie and Willmitzer



**Fig. 5** Quantitative expression analysis of ABA catabolic genes in tuber meristems during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from lateral meristems and the relative fold expression of each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. *CYP707A* gene expression is presented as fold change relative to the initial time point of the storage season. Each gene was analyzed in triplicate. The average and the standard error are reported



**Fig. 6** Quantitative expression analysis of *StCYP707A* genes in periderm during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from periderm. Relative fold expression of each gene was calculated by the method of Pfaffl (2001) using *StCCD* as a reference gene. Transcript abundance of *StCYP707A1* was below the limits of detection. Expression of each gene is presented as fold change relative to the initial time point of the storage season. Values presented are means  $\pm$  SE ( $n = 3$ )

2001). Essential to improvements in dormancy and storage characteristics is an understanding of the processes that control the stop–start growth cycles of potato tuber meristems (Faivre-Rampant et al. 2004a 2004b). Sustained ABA biosynthesis and action are required for both tuber dormancy induction and maintenance (Suttle and Hultstrand 1994). Thus, determining the mechanisms controlling tuber ABA content is crucial to developing a more complete understanding of tuber dormancy control. Using LC–MS, we have shown that after an initial transient increase, the ABA content of meristems and cortex declined during storage by 79% and 55%, respectively (Fig. 1). In periderm, ABA content was unchanged for the initial 97 days of storage but declined thereafter by more than 50%. These results are in agreement with earlier observations (Korableva et al. 1980; Coleman and King 1984; Cvikrová et al. 1994). Similarly, in a study of six cultivars with varying lengths of dormancy, Biemelt et al. (2000) reported that ABA content declined in all varieties during storage but no correlation between final ABA levels and sprouting behavior was observed. Breakage of tuber dormancy with the synthetic cytokinin thidiazuron and heat stress was also accompanied by a decline in ABA content (Ji and Wang 1988; van den Berg et al. 1991). Because ABA levels in situ are determined by the combined rates of synthesis and breakdown, both mechanisms were studied during natural dormancy progression in well-defined potato tuber tissues.

#### Gene expression of the early steps of ABA biosynthesis during tuber dormancy progression

Although recent studies have suggested a possible link between early steps of isoprenoid formation and ABA biosynthesis, regulation of ABA biosynthesis is thought to occur after xanthophyll formation (Nambara and Marion-Poll 2005). In most plant tissues, the rate of ABA biosynthesis is controlled by ZEP and/or NCED enzyme activities which in turn are regulated at the transcriptional level (Nambara and Marion-Poll 2005).

In potato tuber tissues, relative ZEP transcript abundance during storage did not mirror changes in ABA content in any of the tissues examined (Figs. 2–4). No significant increases in ZEP transcript levels were observed in meristems or cortex between days 14 and 27 despite large increases in ABA during the same time period. Conversely in periderm tissues, ZEP transcript abundance increased nearly four-fold between days 14 and 27 while ABA content remained constant. Similarly later in storage (days 97–111), the ABA content in meristems and cortex (but not periderm) again increased significantly while ZEP transcript abundance was constant in meristems but increased in both periderm and cortex.

Others have also noted a lack of correlation between ZEP activity and ABA content in tuber tissues. Transformation of potato with both sense and antisense constructs of ZEP under the control of a tuber-specific promoter, resulted in tubers with elevated or decreased levels of neoxanthin and violaxanthin (both products of ZEP activity) but normal levels of ABA (Römer et al. 2002). Similarly, elevation of neoxanthin and violaxanthin content in tubers through expression of a bacterial phytoene synthase gene had no effect on tuber ABA content (Ducreux et al. 2005).

In many tissues, NCED is considered to be the regulatory enzyme of ABA biosynthesis and its expression correlates with endogenous ABA content (Xiong and Zhu 2003; Nambara and Marion-Poll 2005). We have isolated and characterized two *StNCED* genes that are expressed in potato tubers.

In tuber meristems, *StNCED2* transcript abundance not only exceeded that of *StNCED1* (Table 3) but its expression closely mirrored ABA content with two maxima at 27 and 97 days while *StNCED1* expression increased at 27 days but returned to levels equal to or lower than initial thereafter (Fig. 2). In meristems isolated from tubers chemically induced to exit dormancy, ABA content was highly correlated with *StNCED2* transcript abundance while the *StNCED1* transcript showed little change (data not presented). Collectively, these data suggest that ABA biosynthesis in meristems is mediated by the *StNCED2*-encoded protein. The situation in periderm was less clear. Despite constant or decreasing levels of ABA, expression of both genes was biphasic with two peaks observed at 27 and 111–125 days (Fig. 3). A different pattern was observed in cortex, where expression of *StNCED1* correlated with ABA content while *StNCED2* transcript abundance was highest at the start of storage but declined significantly thereafter (Fig. 4). Thus, although *StNCED1* expression more closely mirrored changes in ABA content in cortex, the extremely low abundance of this transcript in this tissue (Table 3) complicates assessment of its role in ABA biosynthesis. Further experimentation is needed to more clearly define the relative roles for each of these genes in tuber ABA biosynthesis. A similar situation exists in both avocado and *Arabidopsis* where multiple *NCED* genes have been identified that exhibit either tissue-specific or developmentally regulated expression (Chernys and Zeveaart 2000; Tan et al. 2003). In particular during *Arabidopsis* seed development, dormancy induction was dependent on the tissue-specific expression of both *AtNCED6* and *AtNCED9* (Lefebvre et al. 2006). An understanding of the differential regulation of these two NCED genes will provide insight into the developmental and environmental signals that regulate ABA biosynthesis in potato tubers.



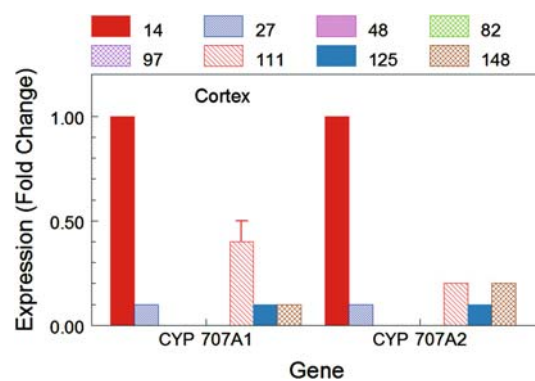
## Expression of the CYP707A genes

Catabolism of ABA to PA via the unstable intermediate 8'-hydroxy-ABA is the first committed step in the principal route of irreversible ABA catabolism in most plant tissues including potato tubers and is catalyzed by *CYP707A*-encoded ABA 8'-hydroxylases (Suttle 1995; Cutler and Krochko 1999). The steady decline in ABA content in tuber tissues (Fig. 1), suggests an increased net rate of catabolism during mid-late storage. This is especially evident in meristems where a >70% decline in ABA content during storage was accompanied by a two-fold increase in the rate of catabolism of [ $^3$ H]-ABA to PA and DPA (Table 2).

Four differentially expressed *CYP707A* genes encoding ABA 8'-hydroxylases have been identified in *Arabidopsis* (Kushiro et al. 2004; Saito et al. 2004). Expression of these genes is up-regulated by dehydration/rehydration, hypersalinity and osmotic stress (Kushiro et al. 2004; Saito et al. 2004). Interestingly, *CYP707A1* and *CYP707A3* are expressed abundantly during the mid-stages of *Arabidopsis* seed development and down-regulated during late embryo development whereas *CYP707A2* accumulates predominantly in dry seed. Also, seeds of *Arabidopsis* T-DNA insertional mutants of *CYP707A2* have higher ABA content and prolonged dormancy (Kushiro et al. 2004).

Three differentially expressed *StCYP707A* genes were detected in potato tuber tissues. All three genes were expressed in meristems during storage (Fig. 5). Although, *StCYP707A3* expression did not change significantly during storage, expression of *StCYP707A1*, the least abundant family member in meristems (Table 3), increased nearly three-fold and five-fold after 97 and 148 days of storage, respectively. In contrast, expression of *StCYP707A2* rose steadily throughout storage reaching a final level ten-fold greater than the initial value. Based on expression levels alone, the increased catabolism of ABA observed in meristems after 91 days could reflect increased transcription of either or both of these genes.

In periderm, expression of *StCYP707A1* was not detected. Expression of both *StCYP707A2* and *StCYP707A3* exhibited a transient 7–60-fold up-regulation between 111 and 125 days of storage (Fig. 6) that coincided with a decline in periderm ABA content during late storage (Fig. 1). Conversely, only expression of *StCYP707A1* and *StCYP707A2* was detected in cortex (Fig. 7) and transcript abundance of both genes declined dramatically during storage rising slightly (but well-below initial levels) after 111 days. The strong down-regulation of these two genes in cortex during mid-late storage suggests that the slow but steady decline in ABA content in cortical tissue was primarily a reflection of reduced biosynthetic capacity rather than increased catabolism.



**Fig. 7** Quantitative expression analysis of *StCYP707A* genes in cortex during postharvest storage. At the indicated times, tubers were transferred from 3 to 20°C 3 days prior to tissue isolation. Real time RT-PCR was performed on total RNA isolated from cortex. Relative fold expression of each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. Transcript abundance of *StCYP707A3* was below the limits of detection. Gene expression is presented as fold change relative to the initial time point of the storage season. Values presented are means  $\pm$  SE ( $n = 3$ )

Thus as is the case in *Arabidopsis* seeds (Kushiro et al. 2004), control of ABA catabolism in different tuber tissues may operate through the action of distinct *StCYP707A* genes. The differential regulation of these *StCYP707A* genes in tuber tissues during storage and dormancy progression may be controlled by developmental cues or by ABA content. In other studies using potato microtubers, both *StCYP707A1* and *StCYP707A3* were strongly up-regulated by ABA application (unpublished results). In either event, ABA catabolism appears to play a central role in the regulation of ABA homeostasis during tuber dormancy.

## Summary

Identification of the site(s) of ABA biosynthesis is essential to more fully understand the relationships between ABA metabolism and development (Nambara and Marion-Poll 2005). All tuber tissues examined expressed the key ABA biosynthetic genes *StZEP* and *StNCED1/2* and at least two of three ABA 8'-hydroxylase genes. This suggests that, during dormancy progression, ABA synthesis and catabolism occurs throughout the tuber. The higher absolute levels of expression of most of the ABA biosynthetic and catabolic genes in meristems relative to the other tissues examined, suggests that meristems are both an ABA target and a principal site of ABA metabolism in potato tubers.

Based on our results, we propose that during tuber dormancy progression and release, endogenous ABA content is modulated by a dynamic balance between biosynthesis and catabolism. Overall during storage, the ability of tuber tissues to accumulate ABA declines as a result of down-regulation of key ABA biosynthetic genes (notably

*StNCED*) coupled with a steady, and sometimes increased, expression of the *StCYP707A* genes. After dormancy release in meristems, the contribution of the catabolic genes becomes increasingly dominant.

The present results suggest that tuber ABA content is controlled in part by both *NCED* and *CYP707A* activities. In tubers, as in other plant tissues (Chernys and Zeevaart 2000; Tan et al. 2003; Koshiba et al. 2004), multiple genes encode these potentially regulatory steps. Using both antisense and RNAi strategies, current research is directed toward elucidating the role(s) of individual gene members in the regulation of ABA levels during tuber dormancy and postharvest storage.

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